EXCYSTATION OF APOSTOME CILIATES IN RELATION TO MOLTING OF THEIR CRUSTACEAN HOSTS. II. EFFECT OF GLYCOGEN[1]

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The apostomes are an order of ciliates symbiotic with Crustacea, being dormant and encysted on their host's exoskeleton for most of their life cycle. All apostomes of the family Foettingeriidae are reported to excyst when their hosts molt (Chatton and Lwoff, 1935). The exuvial fluids trapped in the host's cast-off exoskeleton are the only food of many species (the exuviotrophs). Other genera (the histotrophs) also excyst at the death by injury of their hosts and feed upon the tissue fluids of the corpse.

Excystation is always preceded by an extensive metamorphosis of the encysted stage, the phoront, involving its general body shape, organization, infraciliary pattern and extensive changes in its physiology (Chatton and Lwoff, 1935; Bradbury and Trager, 1967). The metamorphosis prepares the phoront (a non-feeding stage) for the rapid ingestion and concentration of a large volume of food. At the same time the phoront's ciliature is modified and augmented so that later the engorged ciliate (the trophont), swollen to 30 times its initial volume, is still able to swim. The histotrophic phoronts undergo their metamorphosis within a few hours of settling on their hosts. The exuviotrophic phoronts can remain dormant for weeks or months and only metamorphose immediately before the molting of their hosts.

The exoskeletons of Crustacea do not completely seal the animal from its external environment. The exoskeleton is traversed by pore canals and joined by sutures which weaken during the pre-molt period. Food substances—glycogen, lipids, and proteins—build up in the blood and tissues during the premolt stage of the molt cycle (Passano, 1960; Martin, 1965). The possibility therefore exists that some substance (or substances), that increases in concentration in the blood and tissues of pre-molting crabs, leaks out in increasing amounts as the molt approaches and stimulates metamorphosis and excystation of the apostome ciliate. The following experiments were designed to test this possibility.

Materials and Methods

Several hundred hermit crabs (*Pagurus longicarpus*) were maintained in running sea water at the Marine Biological Laboratory at Woods Hole, Mass. The

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pagurids fed on fragments of clam muscle which were always available to them. They appeared active and healthy, molting frequently. All of the molts contained apostome ciliates identified by silver impregnation as *Gymnodinioides* sp. and *Hyalophysa* sp. (probably *Gymnodinioides inkystans* and *Hyalophysa chattoni*). Earlier studies have shown that phoronts occur principally upon the gills (Chatton and Lwoff, 1935; Trager, 1957), and according to observations in connection with our experiments, smaller individuals are more heavily infested than larger individuals.

The appendages of premolting *P. longicarpus* appear grayish, changing to a dark slate gray just before molting. Observed in the dissecting microscope the gray color is iridescent. For several days after molting the new exoskeleton appears reddish.

To prepare for *in vitro* experiments on excystation the pagurids were freed from their mollusc shells by shattering the latter with a jack knife handle. The crab was picked from the fragments and deposited in a petri dish half full of sea water. The area just behind the eyes was pierced with sharp forceps, and the crab was thus pinned to the substrate. The carapace was tested with other forceps to see if the old exoskeleton could be lifted from its surface. Then the carapace was ripped off, and the gills were excised, flush with the surface if possible. They floated free in the water until they could be picked up one at a time with two insect pins and deposited in a drop of sea water on a slide. When all the gills were collected, they were covered by a coverslip and examined immediately in the compound microscope. The extent of infestation was determined and any signs of metamorphosis in the phoronts were noted. Metamorphosis can be recognized by the crowding of food reserves to one side of the organism, the finely granular appearance of the cytoplasm, and the mid-ventral rather than posterior position of the contractile vacuole (Bradbury and Trager, 1967). If molting had progressed so that excysted trophonts were present on the slide, the gills from this crab were discarded. Under the higher magnifications of the compound microscope we noted that freshly excysted trophonts seemed attracted to shreds of flesh attached to gills. While swimming near these shreds of flesh, they became somewhat enlarged and had bright red food vacuoles in their interior.

One to four depression slides were placed in sterile petri dishes on moistened filter paper. All the solutions tested and their controls contained 500 units penicillin and 0.05 mg. streptomycin per ml. Various concentrations of glycogen or of glucose in sea water were put in the concavities of the depression slides, and one or two gills were transferred to each depression. These preparations were examined at intervals with a dissecting microscope for the appearance of moving trophonts. In cases where organisms excysted but did not feed, identification was confirmed with the compound microscope.

In experiments testing blood from alien genera of crabs for stimulation of excystation of phoronts on *Pagurus* gills, blood was taken by hypodermic needle from the articulation between the body and an appendage and mixed in equal volume with a sea-water-antibiotic mixture. Donor crabs were *Carcinus maenas* and *Uca pugilator*. Both green and orange *C. maenas* were maintained in the laboratory. The green form was thought to be far from its next molt. The orange form was believed to be close to molting, although subsequently more than a month passed before any animals molted in the laboratory. *U. pugilator* was considered

TABLE I

Effect of glycogen on experimental excystation of apostome phoronts

"Gray" *Pagurus longicarpus*	Solution	Number of trophonts* after (hours)			
		3	6	8–10	17–20
A†	Sea water		1	1	
	0.25% glycogen		1	1	
	0.12% glycogen		4	4	
	0.05% glycogen		4	6	
B	Sea water	0	0	0	
	0.25% glycogen	0	7	13	
	0.12% glycogen	3	1	1	
	0.05% glycogen	0	0	4	
C	Sea water		0	0	1
	0.25% glycogen		0	0	2
	0.12% glycogen		0	0	1
	0.05% glycogen		0	0	0
D†	Sea water	1		0	
	0.25% glycogen	4		0	
	0.12% glycogen	0		0	
	0.05% glycogen	7		20	
E	Sea water	0		0	1‡
	0.25% glycogen	0		0	0
	0.12% glycogen	0		0	0
	0.05% glycogen	0		0	0

* Blank space indicates no observations made.

† Crabs A, B, C were used in one experiment; Crabs D and E in a later one.

‡ After 20 hours the phoronts remaining on the gills were counted and examined for signs of metamorphosis. Crab D gills in 0.25% glycogen had 27 phoronts, some of which showed metamorphosis; in 0.12% glycogen there were 87 phoronts, many showing metamorphosis; in 0.05% glycogen there 19 phoronts, many showing metamorphosis; in the sea water control were 45 phoronts, none showing metamorphosis. Gills from Crab E in 0.25% glycogen had 12 phoronts, from 0.12% glycogen, 9 phoronts, from 0.05% glycogen 19 phoronts, and in the control 28 phoronts. (No phoront from Crab E showed metamorphosis.)

premolt because of the appearance of an intense blue pigmented patch medially and anteriorly on its carapace.

As a control for the blood and glycogen experiments, gills from each crab were put into separate depressions containing the sea-water-antibiotic mixture.

In some cases Chatton-Lwoff stains were made of gills before the experiment (Corliss, 1953) to see whether any metamorphosis had occurred. In other cases gills at the end of the experiment were stained to see if metamorphosis had begun. Preparing material for staining was so time-consuming that it was not feasible to make Chatton-Lwoff stains a routine procedure in experiments.

RESULTS

Excystation stimulated by blood of other genera of crab

Trager (1957) has shown that *Gymnodinioides* on *Pagurus longicarpus* near molting will excyst more readily in its host's blood than in the blood of *P. longi-*

TABLE II

Effect of blood and glycogen on time of excystation of phoronts*

Solutions	Number of trophonts† after (hours)			
	3	6	9	20
Sea water	0	0	1	30–40
1.0% glycogen	5	5		0
0.5% glycogen	1	1		4
0.25% glycogen	4	8		30–40
0.12% glycogen	4	10		30–40
Blood from orange *Carcinus maenas*				
16%	4	9		30–40
4%	0	3		30–40
2%	0	2		30–40
Blood from green *Carcinus maenas*				
16%	0	0		0
4%	0	0		0
2%	0	0		0

* Solutions were tested on gills from a single gray *Pagurus longicarpus* bearing phoronts in the process of metamorphosis as determined by Chatton-Lwoff silver impregnation. This crab was unusually heavily infested.

† Blank space indicates no observations made.

carpus not near molting. Blood from genera other than the host also seems effective (Trager and Siddiqui, 1963, unpublished observations). In five experiments phoronts from gills of gray *Pagurus longicarpus* excysted in the blood of *Carcinus maenas* and in one case in the blood of *Uca pugilator*.

In the first experiment two engorged trophonts appeared within 11 hours in blood from a green *Carcinus maenas*. *Uca* blood had no effect, and phoronts from the gills of a red *P. longicarpus* tested at the same time were unaffected by blood from either genus.

Experiment 2: Two engorged trophonts appeared within four hours in blood from a green *Carcinus maenas,* but gills from the same crab were not affected by blood from an orange *C. maenas* nor did excystation occur in the sea-water control.

Experiment 3: A single trophont appeared in nine hours in *Uca pugilator* blood from a crab judged near molting. Within 24 hours two other trophonts had appeared. None appeared in the sea-water control.

Experiment 4: Gills from two gray *Pagurus longicarpus* were tested. Chatton-Lwoff silver impregnations of phoronts on Crab A showed no metamorphosis, but many phoronts on Crab B showed metamorphosis, indicating that this crab was probably near molting. Within five hours four trophonts from Crab B gills appeared in the sea-water control. An hour later four trophonts from the gills of the same crab appeared in the blood from an orange *Carcinus maenas*. One trophont from Crab A appeared in blood from an orange *C. maenas*. After 17 hours two trophonts from Crab A appeared in orange *C. maenas* blood and five trophonts from Crab B also appeared in the same blood. Ten trophonts were seen in Crab B's sea-water control.

Experiment 5: Gills from two gray *Pagurus longicarpus* were tested. Silver

TABLE III

Experimental excystation of phoronts from "red" Pagurus longicarpus

"Red" Crab	Concentration	Number of trophonts* after (hours)		
		26–28	40	46
A†	Sea water	0	0	
	0.25% glycogen	2	2	
	0.08% glycogen	0	1	
	0.25% glucose	0	0	
	0.08% glucose	0	0	
B	Sea water	0	0	
	0.25% glycogen	0	0	
	0.08% glycogen	0	0	
	0.25% glucose	0	0	
	0.08% glucose	2	2	
Pooled gills from Crabs C, D, E†	Sea water	0		1
	0.50% glycogen	14		2
	0.25% glycogen	9		10

* Blank space indicates no observation made.

† Crabs A and B were used in one experiment; Crabs C, D, E in a later one.

impregnation of the gills of Crab B showed metamorphosis in the phoronts. In less than four hours one trophont from Crab B gills appeared in blood from a green *Carcinus maenus* and five trophonts in blood from orange *C. maenas*. In 14 hours three trophonts from Crab B gills appeared in blood from green *C. maenas* and six trophonts in blood from an orange crab. Eleven trophonts from Crab B were not present in the sea-water control. A single trophont from Crab A gills appeared in blood from a green *C. maenas* and another trophont appeared in the sea-water control.

In the last two experiments the drawbacks to using relatively high concentrations of blood were especially obvious. The blood mixtures were viscous and cloudy, and the gills were blackened.

It should be noted that excystation of phoronts which had already metamorphosed occurred also in the sea-water controls but generally later than in the blood mixtures.

Effects of glycogen on metamorphosis and excystation

In preliminary experiments gills from grey *Pagurus longicarpus* were placed in 1.0, 0.5, 0.25 and 0.12% concentrations of glycogen and in sea water alone (as always with antibiotics). Within 4 hours trophonts had appeared in 0.12% glycogen only. The results of further experiments with gills from grey *Pagurus* are summarized in Tables I and II. With the exception of Crab E (Table I), where only one trophont appeared altogether, trophonts regularly appeared earlier and in larger numbers in the presence of glycogen. As in the earlier experiments, blood from an orange *Carcinus maenas* was effective, but that from a green one appeared to be inhibitory (Table II).

Especially significant were the results obtained when glycogen was used with

phoronts on gills from post-molt "red" *Pagurus longicarpus*. Whereas excystation had never been observed in such material even when placed in homologous or heterologous blood, and despite the relatively light infestation of such crabs, appreciable numbers of trophonts appeared in the presence of glycogen (and in one case of glucose) (Table III). None of these trophonts fed. They swam about actively for various periods of time and then degenerated and died.

Discussion

The change from phoront to trophont involves two steps, first metamorphosis within the cyst and then excystation. Both of these evidently can be induced *in vitro* by appropriate concentrations of crab blood or of glycogen. It is worthy of note that three different commercial preparations of highly purified glycogen all gave the same results. Of special interest is the fact that a glycogen-sea water mixture induced metamorphosis and excystation of *Hyalophysa* and *Gymnodinioides* in gills of recently molted "red" *Pagurus*. Certainly glycogen must be one of the factors involved in the change from phoront to trophont occurring at the time of molting of the host crab. That it is the only factor seems unlikely, since the numbers of trophonts obtained *in vitro* rarely approached those observed in shed skins under natural conditions. Other obvious factors which might play a role are changes in oxygen tension (this may decrease in the gill chamber shortly before molting) and pH (exuvial fluids are strongly alkaline with a pH around 9). At present we can only speculate as to how a substance like glycogen can have a morphogenetic effect involving extensive rearrangement of previously formed organelles (Bradbury and Pitelka, 1965; Bradbury, 1966), as well as new synthesis.

The occasional excystation which occurred in the sea-water controls from phoronts already metamorphosed, and the still rarer and delayed instances of metamorphosis in the controls, may have resulted from substances exuding from the gill tissue. This might be comparable to Miyashita's (1933) observation of excystation of *Hyalospira* occurring when the cysts were put under a coverslip with fluid squeezed from the host shrimp.

The histotrophic apostomes, which excyst when the host is injured, and those of the genus *Foettingeria* which excyst when the host is eaten (usually by a coelenterate) differ from the exuviotrophic apostomes, dealt with in the present work, in that they undergo step 1, the metamorphosis, almost immediately after encysting on their host. Hence they are ready to excyst, as it were, at a moment's notice, an obvious requirement if excystation is to occur in response to instantaneous events such as injury or predation. The exuviotrophs on the other hand do not undergo their metamorphosis until immediately before their host will molt, in response to altered physiological conditions in the host. One of these effective conditions has now been shown to involve an increased concentration of glycogen.

Summary

To test the hypothesis that a substance or substances normally present in the blood and tissues of pre-molt crabs initiates metamorphosis and subsequent excystation of apostome phoronts, we put excised hermit crab gills bearing *Hyalophysa* and *Gymnodinioides* phoronts in sea-water-antibiotic solutions containing blood

from other species of crabs or low concentrations of glycogen. As a control gills from the same crab were put into the sea-water-antibiotic mixture alone. The glycogen solutions were more effective in inducing both metamorphosis and excystation of the phoronts than the heterologous crab blood. In repeated trials, in which the controls showed no changes, metamorphosis and excystation occurred with glycogen concentrations of 0.12 to 0.5%. This was true even if the phoronts were on gills taken from a hermit crab only recently molted and hence far from its next molt.

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